Regulation of the dnaK Operon of *Streptomyces coelicolor* A3(2) Is Governed by HspR, an Autoregulatory Repressor Protein

GISELDA BUCCA, ZOË HINDLE,† AND COLIN P. SMITH*

Department of Biochemistry and Applied Molecular Biology, U.M.I.S.T., Manchester, M60 1QD, United Kingdom

Received 3 February 1997/Accepted 29 July 1997

Free-living organisms generally respond to an injury caused by exposure to a physicochemical or metabolically stressful agent by inducing the synthesis of a set of proteins, collectively called heat shock proteins (Hsps), which help to prevent or repair damage caused by partially denatured proteins that may or may not have formed insoluble aggregates (15, 19). Some Hsps are molecular chaperones which prevent the aggregation of partially unfolded proteins and help them to fold into their native structures (reviewed in reference 18), while others are proteases, such as Lon and Clp, which degrade unfolded proteins that have formed insoluble aggregates (see, e.g., reference 16). Some Hsps, such as the molecular chaperones Hsp70 (DnaK) and Hsp60 (GroEL), also fulfill crucial roles under normal physiological conditions by assisting in the folding of newly synthesized polypeptides (see, e.g., reference 18). Although these chaperones rank as perhaps the most highly conserved proteins in nature, diverse regulatory mechanisms have evolved in different organisms for controlling their synthesis. In *Escherichia coli* the coordinated induction of the heat shock regulon is achieved by increasing the cellular concentration of two alternative σ factors: σ^32 activates heat shock genes following a mild heat shock (9, 14, 45), while σ^3 activates transcription under conditions of severe heat shock (31, 33). This positive mode of regulation is moderately conserved in Gram-negative bacteria, particularly in the γ subgroup of purple bacteria to which *E. coli* belongs (27). It has recently become clear, however, that many bacteria utilize a repressor/operator system for controlling heat shock genes. The most widespread negative-control system involves the binding of a specific repressor, HrcA (formerly called OrfA, or Orf39), to a highly conserved operator element designated CIRCE (see, e.g., references 3, 4, 32, 35, 37, 44, and 46). Some gram-negative species have recruited both the HrcA/CIRCE and alternative σ factor control systems for controlling different heat shock genes (3), while others have combined both systems for modulating the expression of specific operons (1, 32). With the current exception of the dnaK operons of actinomycete bacteria, the dnaK and groE operons of all gram-positive bacteria studied appear to be regulated by the HrcA/CIRCE system (reviewed in reference 38).

We are studying the regulation of the heat shock response in the model streptomycete *Streptomyces coelicolor* A3(2). Streptomycetes are members of the ancient order *Actinomycetales*, which also includes the mycobacteria, and they are part of the phylogenetically distinct high-G+C gram-positive group. We have characterized the dnaK operon of *S. coelicolor* and studied its regulation (6, 7). The *S. coelicolor* dnaK operon promoter region does not contain a CIRCE element (6), whereas its two groE operons each contain two promoter-proximal CIRCE sequences (12, 13). The dnaK operon encodes four proteins, DnaK, GrpE, DnaJ, and HspR (see Fig. 1). HspR represents a novel heat shock protein which binds to three inverted repeat sequences (IR1 to IR3) in the dnaK operon promoter region (6). There is no sequence similarity between the HspR/IR and HrcA/CIRCE systems, and HspR appears, so far, to be unique to the actinomycete bacteria. We previously speculated that HspR functions as a repressor of the *S. coelicolor* dnaK operon (6). This role is confirmed in the present study. We show that disruption of hspR leads to high-level constitutive expression of the dnaK operon but does not significantly perturb heat shock regulation of the two groE operons. HspR titration experiments were also conducted and provided confirmation that HspR functions as a negative autorepressor. We also provide additional biochemical evidence that HspR directly regulates transcription of the dnaK operon.
MATERIALS AND METHODS

Bacterial strains and plasmids. S. coelicolor A3(2) strain MT1110 (13a) is a SCP1- SCP2- derivative of the prototrophic wild-type strain 1147 (23). Streptomyces lividus 1326 is the prototrophic wild-type strain (23). E. coli XL1-Blue (Str, supE44, lacIq, T3, T10, recA1, endA1, hsdR17 (rK-mK), gyrA96, thi-1, relA1, and lacIq) was used as the general cloning host, and E. coli BL21 (DE3, pLysS) [F-ompT hsdS (rK-mK) g6 lac dcm (A3DE3) P1 lysS (Cm)] (43) was used for overproduction of HspR. E. coli ET1D567 (F- amdm-13: Tn9 der-cm6 hsdMB recF1458 rpsD20: TrirEFnplJ136) (25) was used for propagating plasmid DNA free of dam and hsd methylation. E. coli plasmid pG4.2, which contains the entire dnaK gene and 0.3 kb of the grpE gene of S. coelicolor (7), was used as the source of dnaK probes for Southern analysis and S1 nuclease mapping experiments. E. coli plasmid pG480, containing a 480-bp PstI fragment of pIJ963, was obtained by digestion of the HspR-binding sites on a 200-bp SacII·Nc01 fragment. The latter, encompassing nucleotides –165 to +35 relative to the transcription start site, was used as the template for HspR titration studies; the fragment was first blunt ended and subcloned into the SacI site of pMT3000 (29), a pUC-derived derivative, and then inserted into pMT3010 (29), a multicopy pH101 derivative, as an EcoRI·XbaI fragment to produce pMT3301. Plasmid pGRP17, which contains the 3’ end of grpE and the complete dnaJ and hspR genes (6), was used for the construction of the hspR null mutant. Expression plasmid pT7-7::orfX (a pT7-7 derivative containing an engineered hspR gene [6]) was isolated to isolate the hspR gene for subcloning into the HIS-tagging expression vector pET12b (Novagen). Streptomyces plasmid pMT3010, a multicopy pH101 derivative (29), was used for subcloning the dnaK promoter for HspR titration studies. Plasmids pAM12 and pAM20 (12) were used as the sources of DNA probes for transcripational analysis of groEL2 and groES1 expression, respectively.

Construction of the hspR-disrupting plasmid pMT3302. Plasmid pMT3099, a derivative of E. coli plasmid pMMB696 (as a 1.7-kb BamHI·PstI fragment) was used as a delivery vector for the hspR null mutant. The grpE gene resistance system (grpE) used for disrupting hspR was isolated from E. coli plasmid pG4.2 as a 1.7-kb BamHI·PstI fragment (23, 24). The hspR fragment was blunt ended by treatment with T4 DNA polymerase and inserted into the unique SmaI site of pGPR17, thus disrupting the hspR coding sequence at codon 8. A 2.5-kb BamHI·XbaI fragment, comprising, respectively, 1 kb (upstream from SunI, within dnaJ) and 0.8 kb (downstream from SunI) of chromosomal DNA flanking the inserted hspR segment, was subcloned from this plasmid into the delivery plasmid, pMT3099, to form pMT3302.

DNA isolation, manipulation, and characterization. Procedures used for cultivation and transformation of E. coli strains were carried out as detailed in reference 34. Streptomyces total DNA preparations were isolated by the Kirby method described in reference 23. Standard methods were used for plasmid purification and the manipulation and cloning of DNA (22, 23, 34). For Southern analysis, DNA was transferred to a Hybond N membrane (Amersham) by the rapid-blot method described in reference 42 and fixed by UV irradiation (UV Stratalinker; Stratagene). Radio-labelled probes were synthesized with the Random Primed DNA Labelling Kit (Boehringer Mannheim).

Streptomyces cultivation, heat shock, and transformation conditions, and isolation of RNA. Surface-grown Streptomyces cultures were used throughout this study. Cultures were grown and were induced for RNA isolation. Total DNA was double digested with SacII·NcoI to identify the chromosomally located promoter sequence. In parallel, nine dilutions (ranging from 2·100-fold) were subjected to EcoRI·XbaI double digestion to identify the plasmid-borne dnaK promoter sequence (which was distally flanked by EcoRI·XbaI sites). A copy number of approximately 50 was determined (from duplicate experiments) by comparing the hybridization signals from the respective digests.

S1 nuclease mapping. The following DNA probes were used for assessing dnaK transcription: for dnaK in S. coelicolor, a 2.5-kb BamHI·EcoRI fragment from pAMD20 containing groES and the 5′ half of hspR; and for groES1, the 4.5-kb PstI fragment from pAMD12 containing most of groEL2. To determine, respectively, plasmid-borne transcriptions from dnaKp and groEL2, a 300-bp probe was generated from pMSTM301 by PCR using primer P1 (upstream from dnaKp [6] and a primer (5′-AGGCCACAGGCACGGGCGTGAAG) internal to the 5′ region of the mclC1 reporter gene of the plasmid; the latter primer was radiolabelled at the 5′ end to ensure that only vector-borne transcription was detected with this probe). S1 hybridizations were performed as described in reference 40. DNA·RNA hybridizations were formed at 30°C to 40°C by employing 30 pg of RNA in each reaction, and the hybrids were analysed by either agarose or polyacrylamide gel electrophoresis as described previously (6).

Overproduction of HspR in E. coli. A 550-bp NdeI·ClaI fragment (the latter end digested with Klenow polymerase) containing the hsp coding sequence was inserted into the pT7-7 derivative (digested with NdeI and BamHI; the latter blunt ended). The structure of the amino-terminal coding region was confirmed by sequencing. E. coli BL21(DE3, pLysS) cultures containing pET15b or pET12b were induced by the addition of 25°C (100 mM IPTG) isopropyl-β-D-thiogalactopyranoside (10 mM IPTG) into the cell extracts under denaturing conditions by chromatography on Ni2+–nitrilotriacetic acid resin (Qiagen) according to the protocol provided by the manufacturer. The eluted His-HspR was refolded, after the protein solution was diluted to 50 μg/ml in 8 M urea–100 mM NaH2PO4–10 mM Tris·HCl (pH 8), by dialysis for 4 h at room temperature against two changes of 1 liter of buffer containing 50 mM HEPES (pH 7.5), 100 mM KCl, 10 mM MgCl2, 1 mM EDTA, 10% glycerol, 1 mM diethiothreitol, and 0.5% Nonidet P-40 (see reference 2).

In vitro transcription assays. In vitro runoff transcription assays were carried out as described in reference 10. The dnaK promoter region was isolated as a 380-bp SacII·XaI fragment from plasmid pG480 (GenBank accession no. L46700; nucleotide coordinates, 1 to 383). The 0.95-kb PvuII·KpnI glycerol operon promoter fragment (41) was used as the control template. RNA polymerase was purified as described in reference 21. In the standard 40-μl reaction volume, refolded HspR (ranging from 5 ng to 20 ng) was incubated with 0.1 pmol of template DNA for 30 min at 25°C in 40 mM Tris·HCl (pH 7.9)–10 mM MgCl2–0.6 mM EDTA–0.4 mM potassium phosphate–1.5 mM dithiothreitol–0.75 mg of bovine serum albumin/ml–20% (vol/vol) glycerol. After the addition of 2 pmol of RNA polymerase, incubation was continued for 5 min at 70°C. The reactions were stopped 10 min later by the addition of 10 μl of termination solution (0.5 mg of RNA/ml–2 M sodium acetate [pH 6]). The transcripts were precipitated with isopropanol and fractionated on a 10% urea–6% polyacrylamide gel. MspI-digested pBR322 was used as a size marker.

RESULTS AND DISCUSSION

Construction of an hspR null mutant of S. coelicolor. In order to assess the role of HspR in regulation of the dnaK operon, the hspR gene was disrupted by cloning the hygromycin resistance gene into the unique SacII site at the start of the hspR coding sequence (Fig. 1). The grpe gene of S. coelicolor was transformed with the resulting plasmid, pMT3302, which does not replicate in Streptomyces (see, e.g., reference 20). Generally 1 to 2 transformants/μg were obtained with covalently closed circular DNA, while approximately 100/μg were obtained with NaOH-denatured plasmid DNA. All transformants obtained were doubly resistant to hygromycin and thiostrepton, indicating that in each case the plasmid had integrated by a single homologous recombination event via one of the two chromosomal sequences flanking the hyg gene. With pMT3302, an integration event via the 1-kb segment upstream from hyg would disrupt the dnaK operon at codon 8 of hspR, while integration via the downstream 0.8-kb segment would re-form an intact operon (since the latter fragment is not internal to the transcription unit [see, e.g., reference 11]). Of 10 transformants analyzed by Southern blotting, 2 had integrated via the upstream segment to generate hspR null mutants (designated MT1151 and MT1153) (Fig. 2). The other 8 transformants (including MT1152, which had integrated non-monomatically via the downstream segment; two of the latter hspR integrants, MT1152 and MT1154, were used as controls in this study. The single-crossover integrants MT1151 through MT1154 were found to be highly stable and were therefore used for the transcriptional studies.

Downloaded from http://jb.asm.org/ on November 7, 2017 by guest
Analysis of heat shock gene expression in hspR null mutants. RNA was isolated from surface-grown cultures of MT1151 through MT1154 cultivated at 30°C and from duplicate cultures heat shocked at 42°C for 15 min. In order to directly compare the effect of the hspR mutation on transcription of the dnaK, groESL1, and groEL2 operons, the same four pairs of RNA preparations were subjected to S1 nuclease analysis with each of the operon-specific probes. The results demonstrate clearly that dnaK transcription is derepressed in an hspR null mutant, with the level of transcription at 30°C being comparable to the induced level in heat-shocked cultures (Fig. 2). This indicates that HspR is principally responsible for negatively regulating transcription of the dnaK operon. In contrast, transcriptional regulation of groESL1 was unaffected in an hspR null mutant. Interestingly, the basal level of transcription of groEL2 at 30°C was slightly elevated (approximately two- to threefold) in the hspR null mutant. It is considered that this partial increase in groEL2 transcript level at 30°C is an indirect consequence of disrupting hspR, resulting either from the enhanced synthesis of the DnaK chaperone machine or other, as yet unidentified, components of the HspR regulon. It may be significant in this context that groEL2, but not groEL1, appears to be essential for viability in another streptomycete, Streptomyces albus (39). In a parallel biochemical study, we found that purified His-HspR protein failed to retard templates containing either the groESL1 or the groEL2 promoter region in gel shift experiments, whereas it efficiently retarded a dnaKp template (data not shown). This result is consistent with the above transcriptional analyses, and we conclude that neither of the groE operons forms part of the HspR regulon.

Attempted titration of HspR by cloning HspR-binding sites at high copy number. HspR represses transcription from the dnaK operon promoter. Therefore, if the level of synthesis of HspR is determined principally by the level of transcription from the dnaK operon promoter, then it would follow that expression of the dnaK operon is controlled by an HspR-mediated negative autoregulatory feedback loop. If this is the case, then it would not be possible to deregulate the operon by titrating away HspR by, for example, introducing multiple copies of its binding site into the cell; titration of the repressor would stimulate the production of more repressor and would therefore maintain repression of the operon. This prediction was tested by cloning the three HspR-binding sites from the dnaK promoter region (IR1 through IR3 [6]) into a multi-copy plasmid to form pMT3301 (see Materials and Methods) and assessing the effect of pMT3301 on regulation of dnaK transcription in S. coelicolor hspR null mutants. Transcript levels from two independent hspR1 mutants, MT1151 and MT1153, were compared with those from the hspR1 integrants MT1152 and MT1154. Transcript levels from the dnaK (A), groESL1 (B), and groEL2 (C) operons were determined by agarose resolution S1 nuclease mapping using the same set of RNA preparations. S1 resistant hybrids in each lane are aligned vertically with the respective hybrids generated from the same RNA preparation. Results were extracted from mycelium grown at 30°C (u) and from mycelium heat shocked at 42°C (i). See Materials and Methods for details of the respective DNA probes used in the S1 hybridizations. An untreated sample of each probe fragment (P) was included on each gel.
transcription. In this experiment, two independent \textit{S. lividans} (pMT3301) clones were analyzed, and plasmid copy number was determined from the same cultures used for RNA isolation; copy number was assessed by comparing the relative hybridization signals from the plasmid-borne \textit{dnaK} promoter region and the corresponding chromosomal sequence. Despite their containing approximately 50 additional HspR-binding sites per chromosome, regulation of the chromosomal \textit{dnaK} operon in these clones was unaffected and heat shock regulation of the plasmid-borne \textit{dnaK} promoters was maintained (Fig. 3). (\textit{S. lividans} and \textit{S. coelicolor} are extremely closely related strains, and for the purposes of this study, they are considered the same; their respective \textit{dnaK} operons have an identical restriction map, and heat shock regulation appears identical in both strains.) Comparable results were obtained by Yuan and Wong (44) in a titration experiment with \textit{Bacillus subtilis} which used a CIRCE-containing multicyclic plasmid, and these results provided support for their prediction that the HrcA (Orf39) protein functions as a negative autoregulator of the \textit{dnaK} and \textit{groE} operons of \textit{B. subtilis}.

\textbf{Overproduction and purification of His-tagged HspR protein.} HspR was previously overproduced in \textit{E. coli} by using the T7 polymerase-based pT7-7 system, and HspR-containing cell extracts from induced cultures were used in gel retardation and DNase I footprinting experiments with the \textit{dnaK} promoter region (6). However, the plasmid construct used in those experiments proved to be unstable, and the yield of HspR from induced cells was unsatisfactory. In the present study, \textit{hisR} was subcloned into the more tightly regulated pET15b vector to produce an amino-terminally histidine-tagged product. The His-tagged HspR could be overproduced in \textit{E. coli} to yield approximately 5 to 10\% of total cellular protein (data not shown). Cell extracts containing the His-tagged HspR were shown to specifically retard the \textit{dnaK} promoter fragment, demonstrating that the presence of the \textit{his} tag did not alter the DNA-binding activity of the protein (data not shown). It was not possible, however, to purify the His-HspR directly from the cell extracts by metal affinity chromatography; the protein was not retained on a nickel (Ni-nitrilotriacetic acid) column, and it was considered likely that the amino-terminal His tag was sequestered within the tertiary structure of the protein. Therefore, in order to purify the His-HspR, chromatography was repeated under denaturing conditions (in the presence of 8 M urea), and His-HspR fractions of 90 to 95\% purity were subsequently refolded as described in Materials and Methods. The activity of the renatured His-HspR was assessed by competition gel retardation studies (see, e.g., reference 6) and was shown to bind specifically to the \textit{dnaK} promoter fragment (data not shown).

\textbf{Repression of \textit{dnaK} transcription in vitro by His-HspR.} In vitro transcription experiments were conducted in the presence and absence of His-HspR to assess whether the protein, in isolation, could repress transcription from the \textit{dnaK} promoter. In parallel with the \textit{dnaKp} reactions, a negative-control template was used, containing the glycerol operon (\textit{gyl}) promoter region of \textit{S. coelicolor} (41). HspR was shown to virtually abolish transcription from the \textit{dnaK} promoter (Fig. 4). Although HspR also reduced transcription from the control \textit{gyl} template, the repression ratio was substantially lower. The latter repression is considered to result from interference caused by a degree of nonspecific binding of refolded or partially folded His-HspR. In a separate study, the \textit{dnaK} promoter was used as a control template in analyzing GylR-mediated repression of \textit{gyl} transcription in vitro (21); in that study, purified GylR protein was shown to cause complete repression of transcription from the \textit{gyl} template, but it also repressed, to a much lesser extent, transcription from the \textit{dnaK} template. Although the results from the in vitro transcription experiments cannot alone be used as unequivocal evidence that HspR represses the \textit{dnaK} operon, they are entirely consistent with the results of the \textit{hisR} disruption experiments and with our previous observation that HspR binds specifically to three sites within the \textit{dnaK} promoter.

\textbf{Concluding remarks.} Our studies indicate that the \textit{S. coelicolor} HspR protein represses transcription of the \textit{dnaK} operon
and regulates its own synthesis. Moreover, the transcriptional studies of hspR null mutants indicate that HspR does not regulate the two groE operons of S. coelicolor, although loss of HspR did appear to slightly enhance the basal level of transcription of groEL2.

In low-G+C gram-positive bacteria, such as B. subtilis, the dnaK and groE operons are members of the same regulon, under the control of the HrcA/CIRCE system (see, e.g., references 35 and 44). Although streptomycte dnaK operons are not subject to such control, there is strong circumstantial evidence that their groE operons are regulated by the HrcA/CIRCE system (see, e.g., reference 13). Thus, streptomyctes have recruited two separate repressor/operator systems for controlling the synthesis of the two key chaperone machines. This observation suggests that there is not a strict requirement for a particular stoichiometry of the two systems within the cell, and it is consistent with the current notion that they form part of a complex network for assisting protein folding rather than an obligatory pathway, where the two systems act successively (see, e.g., reference 8). HrcA and HspR share the common function of being negative autoregulators (reference 44 and this work), and their respective genes are contained within heat shock operons which encode molecular chaperones (or co-chaperones). The coinduction of the repressor along with its target structural genes provides a mechanism for rapidly suppressing expression of the heat shock regulon following a burst in Hsp synthesis. It is not yet known how the activity of HrcA or HspR is influenced by heat shock. However, a recent study on regulation of the groESL family of Bradyrhizobium japonicum revealed that the GroEL4 protein negatively influences expression of its own CIRCE-regulated groESL operon, raising the interesting possibility that the GroEL1 chaperonin may control the activity of the presumptive HrcA homolog of that organism (3). In E. coli the synthesis and activity of σ32 is negatively regulated by the DnaK chaperone machine (reviewed in references 9, 14, and 44). This provides a negative feedback mechanism for balancing its own synthesis and that of other Hsps, and it appears that some eukaryotic organisms have evolved a similar control strategy, since Hsp70 has been shown to negatively modulate the activity of HSF, the heat shock transcriptional activator (see, e.g., reference 26). In contrast, dnaK null mutants of B. subtilis are unaffected in regulation of dnaK and groE expression (36). We have circumstantial evidence that the S. coelicolor dnaK gene is essential, since it has proved impossible to disrupt it, despite repeated attempts and the adoption of different strategies (5); it has not, therefore, been possible to assess whether DnaK plays a role in regulating heat shock gene expression in S. coelicolor. We are currently undertaking genetic and biochemical experiments to determine how the activity of the S. coelicolor HspR protein is modulated by heat shock.

In a recent study, Grandvalet et al. (17) exploited the S. coelicolor hspR sequence to isolate the corresponding hspR gene from the taxonomically distant streptomycte S. albus. They disrupted the hspR homolog and concluded, likewise, that HspR regulates dnaK, but not the groE genes, of S. albus.

ACKNOWLEDGMENTS

We thank Bob Beynon, Keith Chater, and Fiona Flett for helpful discussions, and we thank Philippe Mazodier for providing plasmids pAMD12 and pAMD20.

This work was partly supported by a grant from the Biotechnology and Biological Sciences Research Council (United Kingdom), and at the beginning of this work, G.B. was partly supported by a fellowship from the University of Palermo (Palermo, Italy).

REFERENCES


14. Frazer, C. Personal communication.


25. Lydiate, D. J. Personal communication.


